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Effects of Lactobacillus Rhamnosus on dental implant osseointegration in osteoporotic rabbit model

Evaluation of probiotics on dental implant osseointegration

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Abstract
Aim: The effect of Lactobacillus rhamnosus (LR), probiotic bacteria that manage inflammatory disease, on osseointegration is unknown. The study aimed to evaluate the effects of LR administration on osseointegration in experimental osteoporosis induced by ovariectomy.
Material and Methods: Sixteen New Zeland female rabbits were randomly divided into the following groups: SHAM (SH), SHAM and LR (SH+LR), Ovariectomy (OVX), OVX and LR (OVX+LR). Animals in the OVX group were subjected to bilateral ovariectomy to create artificial osteoporosis. After 16 weeks, a titanium dental implant was bilaterally implanted into each tibia of the animals. Animals in the SH+LR and OVX+LR groups were given oral probiotics. All animals were sacrificed and all tibia bones were separated at postoperative 8 weeks after implantation. Cardiac blood was taken to determine biochemical markers. Resonance frequency analysis (RFA) and bone-to-implant contact area (BIC) were administered.
Results: RFA and BIC area were statistically significant for osseointegration in OVX+LR compared with OVX ($p<0.05$). The BIC area in the OVX group was statistically lower ($p<0.05$). ALP, BALP, Trap5b and MPO were highest in OVX groups($p<0.05$).
Discussion: This study concluded that osseointegration may be improved by LR administration in an estrogen-deficient state. The success rate of the implant may be increased by using probiotics in patients with osteoporosis.

Keywords
Biochemistry; Histomorphometry; Lactobacillus Rhamnosus; Osseointegration; Osteoporosis; Probiotics

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Introduction

Bone metabolism is directed by two opposite events: the formation of new bone by osteoblasts and removal of old bone by osteoclasts. The balance between cells found in the same tissue at the same time is important in terms of bone mass. A decrease in bone mass and deterioration in the structure of bone cells may result from some systemic disorders such as osteoporosis [1]. The disorder is more prevalent in women because of estrogen deprivation, which is an important risk factor. It is presented to be responsible for the increase in the incidence of low bone density in postmenopausal women and is associated with tooth loss in them due to this fact [1].

Nowadays dental implant in the treatment of edentulousness is preferred as an alternative treatment instead of traditional methods, and its primary stability is necessary to achieve the osseointegration of implants. Osseointegration, known as the contact between the bone tissue and implant surface, depends not only on the biocompatibility of materials, but also on the health of the adjacent tissue. Dental implant treatment in patients with osteoporosis is considered a risk factor for implant osseointegration since it is characterized by bone loss in addition to alteration of the microstructure of bone. Many studies have shown a positive relationship between bone quality and implant success [2-4]. It is known that estrogen deprivation in women accelerates osteoclastic activity and positively correlates with bone destruction [5-7]. August et al. reported that the success rate of maxillary implants in postmenopausal women receiving estrogen is twice as high as those who do not receive it [8]. Although there are many treatments to prevent bone loss for the disorder, treatments are not always successful. For this reason, the use of dental implant treatment in patients with osteoporosis is a possible contraindication.

The use of probiotics to maintain oral health is a new field of research. Although there are some common views on the effects of microbiota on bone, there are few studies on this subject. As the intestinal system plays a key role in the metabolism of calcium and vitamin D, probiotics have the potential to regulate bone health. The effect of microbiota on bone is described in the form of nutrient uptake (calcium and phosphate), immune regulation and the production of small molecules such as direct serotonin or estrogen-like molecules. Studies have shown that inflammation in the bone marrow occurs during intestinal inflammation and ovariectomy [9,10]. Mc Cabe et al. reported positive effects of probiotics on cytokine release in rats in addition to an increase in volume and mineral density of the bones displayed by micro-CT in their study [11].

Current studies have investigated the oral and systemic effects of various probiotic bacterial species. One of the widely used probiotics, the gram-positive anaerobic bacterium Lactobacillus rhamnosus (LR), reduced gut inflammation and improved barrier function of the intestine [12]. The bacterium, as a new avenue in the management of postmenopausal osteoporosis, inhibits osteoclastic activity [13]. LR has been shown to accelerate bone calcification and regulate osteocyte formation, but the effects of bone healing around the implant are still unknown [14,15].

In the present study, we have hypothesized that osteoporosis affects the establishment of osseointegration, and LR-administration has a positive effect on bone healing around the implant material in a rabbit model.

Material and Methods

The study was approved by the Local Ethics Committee of Animal Experiments in Sivas Cumhuriyet University. A total of 16 adult female healthy New Zealand rabbits (Harlan, Zeist, The Netherlands), aged 6-9 months and 2,5-3 kg body weight were maintained individually in stainless steel cages in a standard animal facility, and the room temperature remained at 21–24°C with 40–60% relative humidity and a 12-h light-dark cycle. The animals were fed ad libitum. After the quarantine, the animals were randomly divided into SHAM (SH), SHAM and LR (SH+LR), Ovariectomy (OVX), OVX and LR (OVX+LR). The animals were fasted for 24 h prior to operation. Ovariectomy was performed under general anesthesia (Xylazine 25 mg/kg and ketamine 50 mg/kg). In the SHAM-operated group, rabbits were also subjected to the same surgical procedure with the removal of an equivalent amount of fat tissue instead of ovaries (n=8). To create an artificial osteoporosis model, an ovariectomy operation was performed on the other animals under general anesthesia, and bilateral ovaries were removed completely with a midline incision. The rabbits were given 2 daily doses of 50 mg/kg ceftriaxone for 4 days for prophylaxis. It was waited for 16 weeks for the osteoporosis model that requested to be created artificially.

The implant surgery was performed at the end of the 16th week, and only one implant was implanted in each tibia of all subjects. Surgical procedures were performed under general anesthesia with intramuscular injection of pentobarbital sodium (50 mg/kg). The titanium implants designed hybrid features RBM (resorbable blast media) surface structure of 3.3 mm diameter and 7 mm length (Implance, Trabzon, Turkey) were performed bilaterally to the proximal region. Implants were fixed to the tibia by adhering to the surgical procedure and primary stability was obtained. Following intramuscular antibiotic (50 mg/kg Ceftriaxone) and analgesic (4 mg / kg Carprofen) injections, an antiseptic solution was applied.

To ensure adequate intake of the bacteria, half of the SHAM and OVX groups were gavaged 1 drop (1x109 cfu/ml) of probiotics (Lactobacillus rhamnosus, Maflor, Mamsel Drug Industry, Istanbul, Turkey) per day until their sacrification [16]. Probiotic drops were prepared and applied according to the company instructions by mixing them with water (300 µl). Eight weeks after implant surgery, all animals were killed with a lethal dose of pentobarbital sodium (100mg /kg). The tibial bones containing implants were bilaterally removed.

RFA values were calculated for each implant via an sstell device (Osstell Mentor, Integration Diagnostics AB, Gothenburg, Sweden) as implant stability quotient (ISQ) values. ISQ values were recorded after implantation and when the animals were sacrificed, 8 weeks postoperatively.

Tissue blocks (implants and surrounding tissue) of each animals' tibias were collected and the specimens were dehydrated in a graded series of ethanol rinses and embedded in a glycol methacrylate resin (Technovit 7200 VLC, Kulzer, Wehrheim, Germany). The specimens were then trimmed along the longitudinal axis of the implants, and the BIC distance of the section images transferred to the computer was measured using an image analysis program (Analysis LS Research, Version 5.0, Olympus Soft Imaging Solutions). The BIC distance in all groups was automatically calculated by measuring the total

length of the BIC areas in the new bone formed at the top of the most basal part of the implant and following bone boundaries adjacent to the implant using the multi-line marking tab in the computer program in μm unit.

Cardiac blood was taken to determine the levels of markers for osteoblastic and osteoclastic activity. Rabbit bone alkaline phosphatase (BALP), rabbit myeloperoxidase (MPO), rabbit osteocalcin (bone gla protein) (OT/BGP), rabbit collagen type I (Col I), rabbit tartrate resistant acid phosphatase 5b (TRAP5b) were spectrophotometrically searched through ELISA method with commercial agents according to their methods described by them in the blood serum of the animals.

SPSS software program (version 22.0 for Windows; SPSS, Chicago, IL) was used for statistical analysis. The data of RFA, BIC percentages, ALP and COL Type I were statistically analyzed by use of statistical Kruskal- Wallis non parametric test. Wilcoxon Signed- Rank test was used for paired data from initial and final measurements of ISQ values. One-Way ANOVA, a parametric test, was used as a statistical method for BALP, MPO, OT / BGP and TRAP5b because of normal distribution. The obtained data were expressed as means ± standard deviations. Differences were considered as statistically significant at $p < .05$

Results

ISQ values increased in all groups at the 8th week (Table 1). The ISQ values in the SHAM groups detected after implantation were statistically significantly different from the OVX groups ($p<0.05$). After implantation, while the mean ISQ value of the OVX group increased from 64.061 ± 4.207 to 68.262 ± 4.176, this value for the OVX+LR group increased from 63.125 ± 3.981 to 71.264 ± 2.019, and this difference between the groups was statistically significant ($p<0.001$).

The bone implant contact (BIC) area was shown in Figure 1. BIC area was evaluated for both groups, and sections were evaluated (by F.G.). The percentage of %BIC was defined as the ratio of the proportion of the dental implant length in direct contact with newly formed bone tissue and the total length of the implant adjacent to native bone (Figure 1a, b, c, d). The sections were evaluated at least three threads in this process. In the OVX group, it was statistically decreased (35.36 ± 6.11) (Figure 1c). The highest proportion of BIC was determined in SHAM+LR (45,68 ± 3,99) compared to others. But there was no difference between SH, SH+LR and OVX+LR groups (Table 2)

Eight weeks after implant surgery, new bone tissue was observed around the implants. Osteoblasts and osteoid tissue were observed in all histological sections (Figure 2). The organized bone tissue and already immature bone tissue were observed in the sections. The bone tissue formed around the implants was healthy. Bone tissue formations were observed in both group samples. The newly formed bone tissue was observed as more regular and organized in the probiotic used groups (Figure 2b, d). It was observed that there were intense osteocytes in both group samples (Figure 2a, b, c, d).

Osteoclastic serum markers ALP (U/ml), BALP (mIU /ml), TRAP5b (μU/ml), COL Type I (ng/ml) and MPO (ng/ml) were higher in the OVX group (Table 3). BALP, TRAP5b and MPO values belonging to OVX groups were statistically significant ($p<0.05$). When compared with others, the data belonging to COL Type I and MPO were higher in the OVX groups, but the difference was not statistically significant ($p> 0.05$). While the highest mean in the TRAP5b serum marker was obtained in the OVX group, the differences obtained in the binary comparisons were found statistically significant ($p<0.05$).

Table 1. ISQ values of the groups at different times after implant surgery

	SH Mean ± Sd	SH +LR Mean ± Sd	OVX Mean ± Sd	OVX+LR Mean ± Sd	
ISQ Values (Immediately after implant Surgery)	68.176 ± 3,807	65,38±1,94	64,061 ± 4,207	63,125 ± 3,981	P=0.003
ISQ Values (8^{th} week)	71.1 ± 3.18	72,941 ± 2,709	68,262 ± 4,176	71,264 ± 2,019	P=0.001*
	P<0.001*	P<0.001*	P<0.001*	P<0.001*	

*p<0.05 : Statistically Difference SD: Standard Deviation

Table 2. Bone – to – implant contact area values of the groups

Groups	SH Mean ± Sd	SH +LR Mean ± Sd	OVX Mean ± Sd	OVX+LR Mean ± Sd	p
BIC Area	45,25±11.63	45,68 ± 3,99	35,36 ± 6,11*	42±14.06	P<0.001*

*p<0.05 : Statistical Difference SD: Standard Deviation

Table 3. Biochemical markers related to the groups

Serum Bone Markers	SH Mean ± Sd	SH +LR Mean ± Sd	OVX Mean ± Sd	OVX+LR Mean ± Sd	p
ALP(U/ml)	0.0463±0.002	0.0367±0.006	0.0972±0.008	0.0920±0.009	0.005*
BALP (mIU/ml)	0.1759±0.004	0.1751±0.001	0.1863 ± 0,005	0.1831±0.003	0.007*
OT/BGP(ng/ml)	0.0159±0.00	0.0159±0.001	0.0152±0.00	0.0154±0.00	0.456
TRAP5b (μU/ml)	687.39±182.71	509.56±291.08	1291.11±190.06	1125.19±259.34	0.002*
Col TYPE I (ng/ml)	1.5167±0.21	1.5008±0.132	1.6172±0,014	1.5838±0.037	0.058
MPO (ng/ml)	1,4340±0,009	1.4476±0.008	1.4675±0.014	1.4596±0.014	0.012*

*p<0.05 : Statistical Difference SD: Standard Deviation

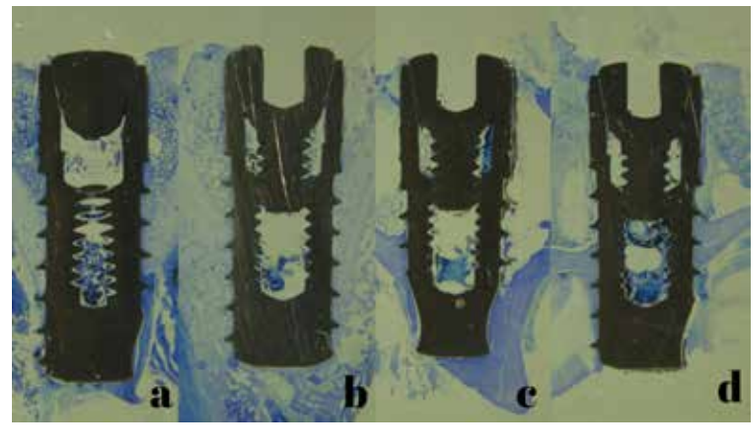

Figure 1. Histologic sections of different groups at 16th wk after implant surgery belong to rabbit tibias (original magnification x10) (Fig.1a,b,c,d). In the SH groups there showed regular bone tissue around implant material (Fig.1a and b). In the OVX groups it was seen that irregular structure in sections (Fig.1c and d). The SH groups showed the bone-to-implant contact and a great quantitiy of bone matrix formation representative histological images. BIC area was lower the OVX groups than the others and this difference showed statistically significance (Fig.1c). Newformed bone tissue was greater shown in the OVX+LR than OVX. Bone density around the implant increased in the groups taking probiotics.
Δ mature bone tissue around the implant material ☐ irregular and great porosities in bone tissue

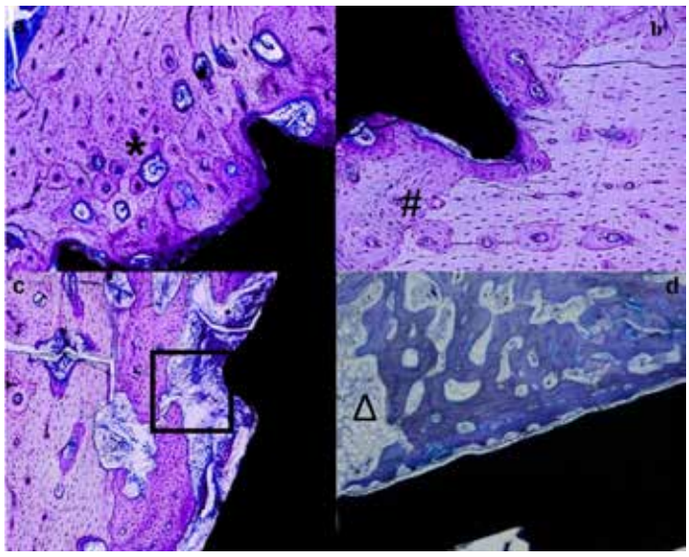

Figure 2. A minimum gap formation was shown between bone and implant surface in SH groups. In contrast, in the OVX groups non-mineralized tissue was observed. The SH groups showed better maturity than the OVX groups (Fig 2a,2b) Osteoblasts and osteoclasts are visible all figures. But bone tissue quality of SH+LR groups appears to be better than the others. Attachment was clearly better in SH groups than OVX groups. Histologic sections showed that new bone was formed around all sections. In the OVX groups taking LR administration not only enhance the osseointegration, but also has mature bone compared with OVX group implants (Figs 2c,2d). Bone tissue around the implants in OVX groups was less intense density. The newly formed osteocytes in the OVX groups are smaller, round and disorganized than the osteocytes in the old bone areas *osteocytes and osteoblasts #mature bone formation ☐ osteoblasts organized near old bone tissue Δ disorganized bone tissue

Discussion

Osteoporosis is a common disease among elderly, especially postmenopausal women, characterized by a decrease in bone mass and microarchitectural changes in bone. As a result, these changes lead to increased bone fragility, and the risk of fracture increases [18]. Postmenopausal osteoporosis is a type of osteoporosis that occurs due to decreased ovarian function and blood estrogen level.

Ovariectomy is the most common method to create an animal model of postmenopause [19]. In the literature, there are many animal models (rat, mice, dogs, cats, etc.) used for the osteoporosis model by removing ovaries. The rabbit model was preferred in the current study because of reaching skeletal maturity at about 6 months, faster bone turnover than in other rodents and primates, and induction of significant bone loss within a short period of time [20]. It was reported that osteoporotic deterioration as a negative effect of ovariectomy appears after about 12 weeks [21]. Wanderman et al. noted that bone mineral density in the ovariectomized rabbit's femur decreased significantly at 16 weeks [22]. It can be assumed that ovariectomized animals were affected by estrogen deficiency during the osseointegration period since the 16-week term, which was evaluated in the current study. After implant surgery, new bone formation can appear around implants at 15 days, and the osseointegration processes can be completed at 8 weeks. Therefore, it was waited for 8 weeks for complete osseointegration.

Rabbit tibia bones have been widely used for the placement of titanium implants in osseointegration. There are various methods to evaluate bone integration around implants. In the present study, histomorphometry and RFA were preferred as objective measurement methods.

According to the results of histomorphometry, ovariectomy was shown to negatively affect the osseointegration process. The sections belong to the OVX groups showed irregular bone structure and high porosity compared the SH groups. Administration of LR appeared to enhance the BIC of implants in the OVX groups. Lugero et al. stated that their histomorphometric study compared the SH and OVX groups at the 8th week of osseointegration process and found a significant difference between the groups in terms of bone formation around the tibia cortical bone thickness, trabecular volume, mineral apposition rate, and titanium implants [23]. A clinical study on healthy, osteoporotic and osteopenia patients also suggested a positive correlation between primary implant stability and bone density [20].

It has been stated that there is a significant relationship between decreased bone quality and primary stability of the implant in-vivo researches [2,3,4]. According to RFA measurements in the OVX groups, ISQ values were lower than in the SH groups. It was thought that estrogen deficiency can negatively affect bone quality as well as primary stabilization of the implant. LR administration in SH and OVX groups was significantly higher than in the OVX group. It may be concluded that LR administration to bone quality and primary stability of the implant. Similar to the current study, in their clinical study comparing primary stability values, it was found that the RFA value was lowest in the OVX groups although the RFA value was highest in the sham groups and the difference was statistically significant.

The modeling process of bone is a condition managed by osteoblast and osteoclast activity. Osteoclastic markers such as ALP, collagen type 1 play an important role after the placement of implants [25]. Similar to the current study, Du et al. reported that estrogen deprivation adversely affects osteocyte morphologies. In their study, ALP, OC, collagen type 1 and TRAP levels were found to be significantly higher in the OVX group and determined as an important indicator of bone model in the early recovery period [7].

There are many studies using different antiresorptive and anabolic agents, which show that osteoporosis affect elderly population by reducing the contact area of bone implant and causing implant failure due to bone structure deterioration. In oral microbiology, the use of probiotics indirectly affected all bone parameters, which can be found in the literature. Studies have shown that microbiota acts on the bone through the host's immune system by regulating osteoclastogenesis [11,14] As the probiotics selected in this study were Lactobacillus and applied during osseointegration, the data obtained from this study support the literature. Britton et al. have reached the conclusion that oral administration of Lactobacillus bacteria (1x109 cfu/ml probiotics in 300µl water) is effective in preventing bone loss and affect serum markers [16]. Liquid probiotic supplements selected in this study were applied as 1 drop every day during

the experiment, as a drop included 1x109 live microorganisms. Probiotics can regulate the gut microbiome (composition and activity), increase barrier function, and decrease intestinal inflammation, resulting in several local and systemic responses, such as (1) reduced inflammation in the gut, blood, and bone, (2) increased metabolite levels such as short-chain fatty acids (SCFA), which can enhance calcium absorption and signal locally in the gut and in the bone increased bacterial secreted factors, (3) and intestinal hormones such as incretins and serotonin that are known to regulate bone density. Finally, the signals result in decreased osteoclast activity and/or increased osteoblast activity leading to enhanced bone density, structure, and strength. However, how this mechanism works is still not fully elucidated.

The results of our study showed improvement in implant osseointegration due to administration of LR in an estrogen-deficient state of bone. Anti-osteoclastic and anti-inflammatory effects of LR could explain this process.

Oral administration of LR has been shown to improve the osseointegration of dental implants that were implanted in the tibial bone of osteoporotic rabbitts. This assumption may be supported by clinical studies assessing the success of dental implants applied to patients.

Scientific Responsibility Statement

The authors declare that they are responsible for the article's scientific content including study design, data collection, analysis and interpretation, writing, some of the main line, or all of the preparation and scientific review of the contents and approval of the final version of the article.

Animal and human rights statement

All procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. No animal or human studies were carried out by the authors for this article.

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Conflict of interest

None of the authors received any type of financial support that could be considered potential conflict of interest regarding the manuscript or its submission.

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